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Understanding the mechanisms involved in the progression from carcinoma *in situ* to metastatic disease is one of the most important problems in breast cancer research. To this end, studies by our group and others have implicated a critical role for the α6 integrins in breast cancer progression. For this reason, it is essential to elucidate the mechanisms by which these integrins contribute to breast cancer progression. This IDEA Award is based on the hypothesis that the α6 integrins regulate expression of VEGF, a growth factor that is essential for tumor survival and angiogenesis. In Year 2 of this Award, we have made considerable progress in validating this hypothesis and we have established that the α6β1 integrin is necessary for the activation of HIF-1α, a transcription factor that regulates VEGF expression in breast carcinoma cells.

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Table of Contents

Introduction: Understanding the mechanisms involved in the progression from carcinoma *in situ* to metastatic disease is one of the most important problems in breast cancer research. In simple terms, progression to metastatic disease requires that breast carcinoma cells acquire the abilities to invade, stimulate angiogenesis and survive in non-breast tissues. Recently, progress has been made in identifying specific molecules that contribute to each of these critical events. These molecules include growth and angiogenic factors, as well as their corresponding receptors, and cell adhesion molecules. The challenge ahead is to understand how these molecules cooperate and interact to promote progression. Studies by our group and others have implicated a critical role for the α6 integrins in breast cancer progression (1) (2-4) (5). The goal of this IDEA proposal is to define the mechanism by which an integrin, such as the α6 integrins contributes to the survival of metastatic breast carcinoma cells. We postulated that these integrins regulate the expression of HIF-1α, a key regulator of VEGF expression and angiogenesis in breast and other cancers. During the second year of this grant, we have made considerable progress that substantiates our hypothesis and completing our statement of work. We developed an effective RNAi strategy for inhibiting the expression of α6 integrins in breast carcinoma cells and used this approach to demonstrate that these integrins are necessary for breast carcinoma survival. Importantly, we have also obtained key data that the α6 integrins are essential for the transcriptional activation of HIF-1α.

Body:

Development of an effective RNAi strategy for inhibiting expression of α6 integrins in breast carcinoma cells: The use of RNAi enables us to reduce expression of the a6 integrins specifically and to assess the consequences on breast carcinoma function. To this end, we designed oligonucleotides for this purpose and expressed them in MDA-435 cells. As shown in Fig. 1 below, this approach resulted in a significant decrease in the expression of the α6β1 integrin but not of the α5β1 integrin. The success of this approach allowed us to use it in subsequent experiments aimed at understanding the contribution of α6β1 to VEGF transcription and breast carcinoma survival.

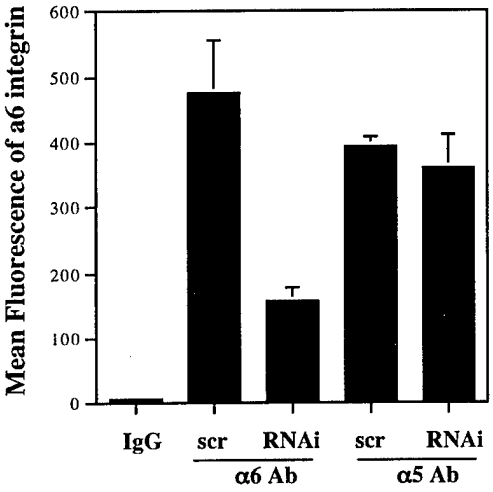
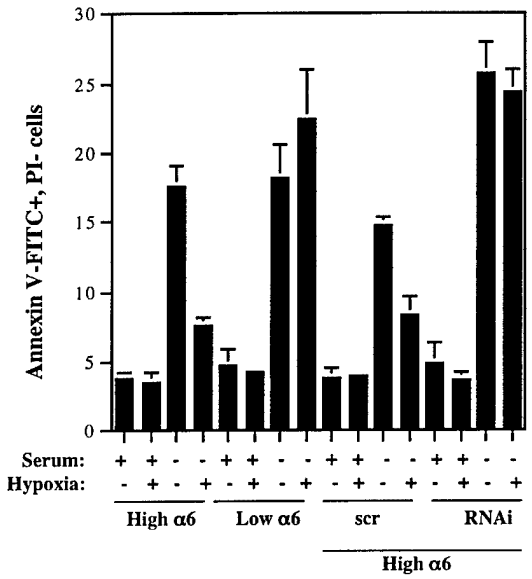


Figure 1: Use of RNAi to Reduce α6β1 Integrin Expression in Breast Carcinoma Cells. Oligonucleotides were designed that inhibit expression of the α6 integrin subunit. These oligos, as well as a scrambled control sequence, were transfected into MDA-435 cells. After 72 hrs, the surface expression of the α6 and α5 integrin subunits was assessed by flow cytometry. Note that the expression of α6 but not α5 is reduced substantially.

Loss of α6β1 Integrin Expression Increases Apoptosis of Breast Carcinoma Cells in Stress Conditions: To examine the hypothesis that α6β1 integrin promotes breast carcinoma cell survival under stress condition, we incubated MDA-435 cells in either normoxia or hypoxia in low serum (0.5% FBS) for 24 hours and then measured annexin –FITC staining (Fig. 2). Serum deprivation dramatically increased apoptosis up to 4 fold while presence of 10% FBS protects cells from apoptosis regardless of α6 integrin level (Fig. 2). However, in high α6 clone, hypoxia reduced the apoptotic level approximately 50% suggesting that hypoxia acts as a survival factor (Fig. 2). Hypoxia driven cell survival against serum starvation is α6β1 integrin dependent because low α6 clone and α6 RNAi treated MDA-435 cells remained highly apoptotic under hypoxia (Fig. 2).



3. The α6β1 Integrin Regulates HIF-1a Activation in Breast Carcinoma Cells: Since HIF-1 has been reported to play a major role in target gene transcription under hypoxia, we assessed the induction of HIF-1α expression by hypoxia in clones of MDA-435 cells that exhibited either low or high α6β1 levels. As shown in Fig. 3, HIF-1α induction was comparable on these clones, suggesting that the α6β1 integrin is not involved in the regulation of expression or stabilization of HIF-1α.

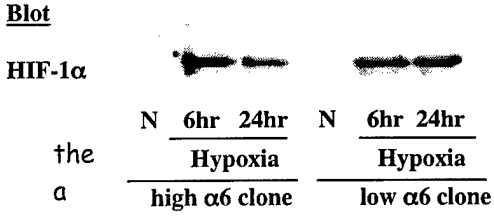


Figure 3. HIF-1α Induction by Hypoxia is Independent of α6β1 Integrin Levels. MDA-435 breast carcinoma cells that exhibited either high or low expression of α6β1 were incubated in either normoxia or hypoxia for indicated times. Detergent extracts were blotted with HIF-1α specific Ab.

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To assess HIF-1a activation, we monitored the association of HIF-1 with its co-activator, p300 (Fig. 4). HIF-1 binding to HRE itself is not enough to activate target gene transcription and HIF-1 association with p300 is required to recruit DNA pol II complex to initiate transcription. Therefore, the association of HIF-1 with p300 is an indicator of its activation. The association of HIF-1 with p300 was maximal level at 6 hour and gradually declined in high α6 clone (Fig. 5B). In the low α6 clone, the association of HIF-1 with p300 was barely detectable (Fig. 5B). The level of p300 expression remained constant in normoxia and hypoxia in both the high and low high α6 clones (Fig. 5A and B). The results indicate that, although the induction of HIF-1a is comparable during hypoxia regardless of α6β1 integrin level, α6β1 integrin is involved in HIF-1 activation.

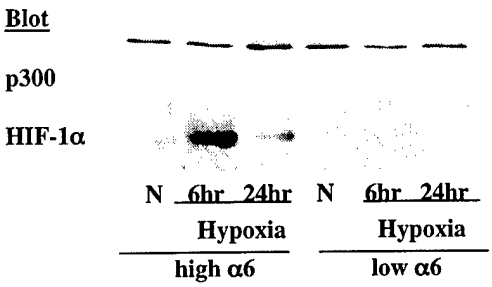

Figure 4. The α6β1 Integrin is Necessary for HIF-1α Activation in Breast Carcinoma Cells. MDA-435 breast carcinoma cells were prepared as described in Fig. 3, immunoprecipitated with a p300Ab and blotted with a HIF-1α Ab.

Finally, to confirm the role of HIF-1α in VEGF trsancription under hypoxia, we used a HIF-1a dominant negative mutant that blocks HIF-1α binding to HRE. Expression of two different dominant negative HIF-1α mutant blocked activation of VEGF transcription as shown by luciferase reading (Fig. 5), indicating that HIF-1α and not other transcription factors, is playing a major role in VEGF transcription under hypoxia. Taken together with the data shown in Figs 2-4, these results provide direct evidence for the involvement of α6β1 in HIF-1α-mediated VEGF transcription.

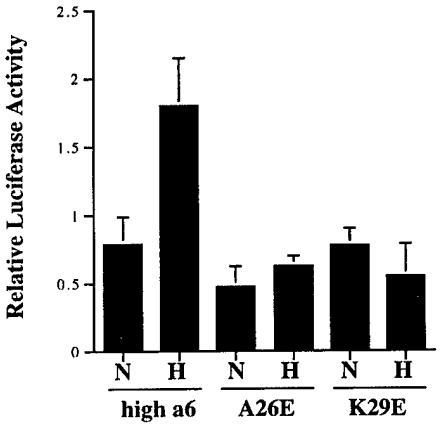

Figure 5. VEGF Transcription is HIF-1α Dependent in Breast Carcinoma Cells. MDA-435 cells that expressed high levels of the α6β1 integrin were transfected with either a control vector (high α6) or one of two dominant negative HIF-1α constructs. Cells were maintained in either normoxia or hypoxia for 24 hrs and VEGF transcription was assessed by luciferase assay.

Key Research Accomplishments:

- Developed effective RNAi strategy for inhibiting expression of α6 integrins in breast carcinoma cells.
- Demonstrated the importance of α6β1 integrin for the survival of breast carcinoma cells in hypoxia using this RNAi strategy.
- Established that α6β1 regulates the activation of HIF-1α in hypoxia but not its expression.
- Provided evidence that VEGF transcription in breast carcinoma cells is HIF-1α dependent.

Reportable Outcomes:

A major manuscript that includes the results described in this report is currently in preparation and will be completed during the first part of Year 3 of this award.

Conclusions:

During Year 2 of this award, we have completed a significant amount of original Statement of Work. Most importantly, the data we have obtained validate the central hypothesis of our original proposal. In the remaining year of the award, we will complete the remaining items in Tasks 2 and 3 of our SOW.

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Appendix:

None